THE USE OF AFFINITY CHROMATOGRAPHY IN THE STUDY OF PROTEIN FOLDING

Christian B. Anfinsen

Department of Biology
Johns Hopkins University
Baltimore, Maryland

Much of the current research in the field of synthetic vaccines is based on the assumption that fragments of antigens --for example, fragments of viral coat proteins or toxins-- can, upon attachment to a neutral carrier structure and injection into patients, induce the synthesis of antibodies that will recognize and immobilize the pathogenic agent. Antibodies recognize native conformations of protein determinants ("conformational determinants") and also primarily random peptide fragments ("sequential determinants") (1). It becomes important to determine the extent to which the latter, random structures can take on the "native format" that such fragments exhibit when part of the native protein structure. Only by such involvement in an equilibrium between the random and properly folded conformation would the fragment serve as a meaningful antigen in vaccine production.

A number of years ago (2) we showed that a fragment of hen's egg white lysozyme could be synthesized, attached to a carrier module, and be injected into animals with the production of antibody that would react with, and inactivate the original enzyme molecule. The synthetic material was, in a sense, a "vaccine" against lysozyme. As will be seen in Figure 1, this antigenic lysozyme "loop" was stabilized in a suitable conformation by a disulfide bridge. Peptide fragments without such stabilization would tend to occupy a large set of random conformations, very few of which would be likely to be recognized by an antibody formed against the native, parent protein molecule.

We have used affinity chromatography as a tool in the study of the interaction of peptides with antibodies ($Antibody_N$) raised against the native protein. By the attachment

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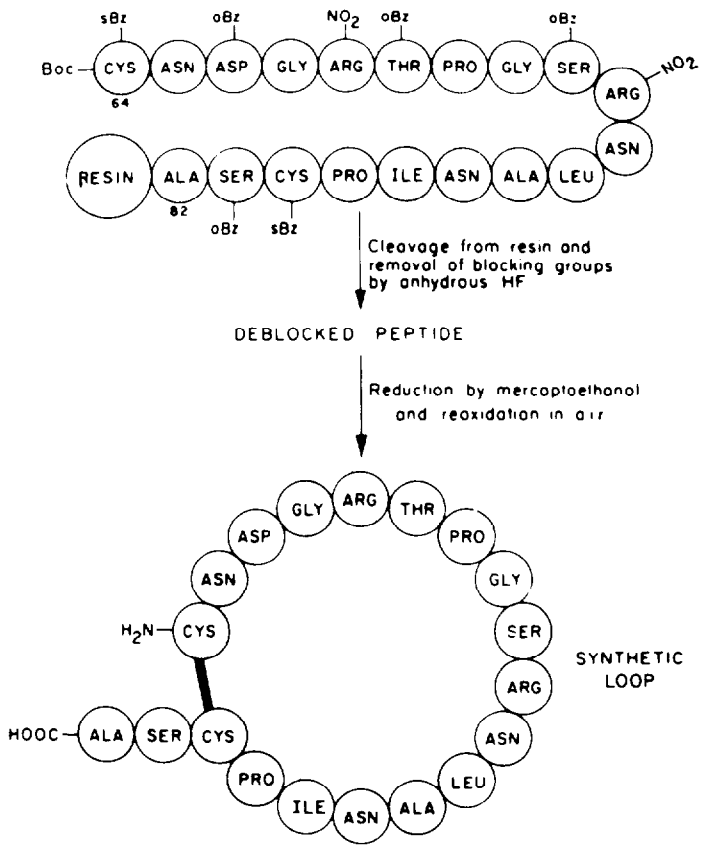

FIGURE 1. The solid phase synthesis, deprotection and ring closure of a peptide "loop" in hen's egg white lysozyme.

of fragments of the protein chain to a Sepharose supporting backbone, subfractions of the mixed Antibody$_N$ population can be isolated. The isolation of such monospecific antibodies provides reagents that are no longer precipitating. Thus, one can deal quantitatively with antigen-antibody reactions in solution, and measurements are made more easily and more accurately. The binding to such columns of monospecific antibodies already suggests that fragments of the protein molecule, which appear to be totally lacking in structure as determined by the usual physical measurements, do indeed occupy ordered structure for at least a small portion of their lifetime. Thus, a peptide appears to "flicker" between the random form and the form that the peptide occupies when it is part of the

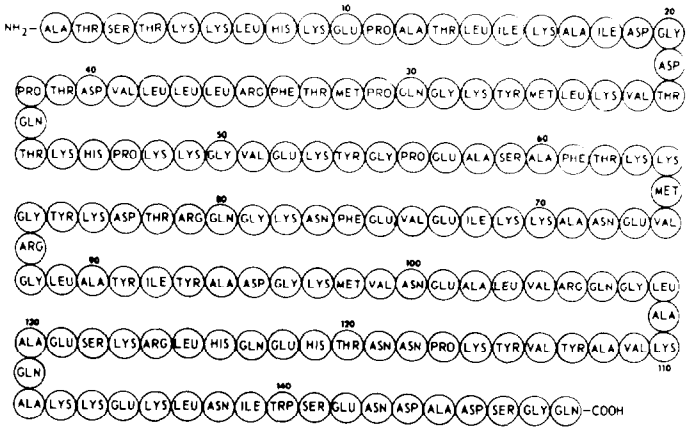

FIGURE 2. The amino acid sequence of staphylococcal nuclease.

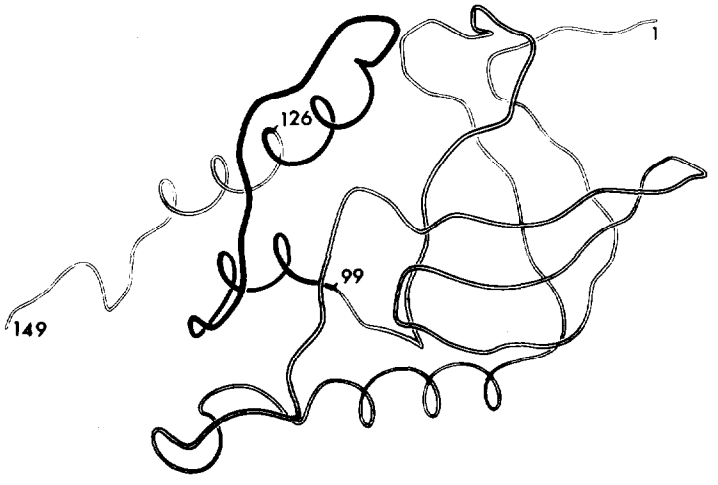

FIGURE 3. Drawing of the three-dimensional structure of staphylococcal nuclease.

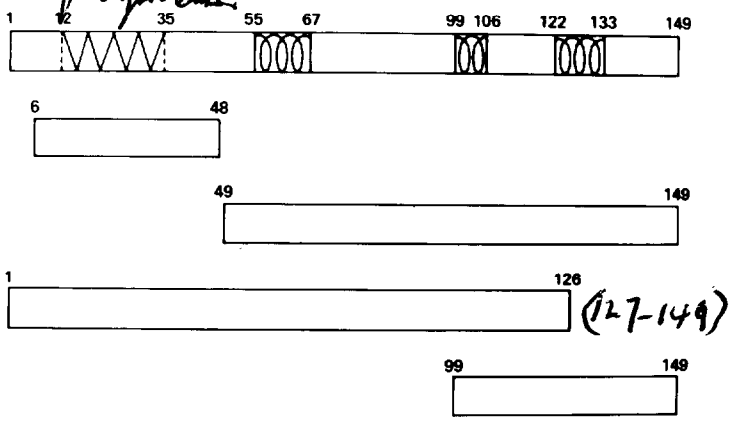

FIGURE 4. Some fragments prepared from staphylococcal nuclease. Top: location of pleated sheet and helical sections of nuclease. Fragments labelled 6-48 and 49-149 are produced by limited trypsin digestion. Peptide 1-126 (and the fragment 127-149, not shown) are obtained by trypsin digestion of trifluoroacetylated nuclease with subsequent removal of the TFA protecting groups. Peptide 99-149 is one of five fragments produced by cyanogen bromide digestion of the protein.

native protein structure. Guided by its amino acid sequence, it can occasionally assume its conformation in the intact parent molecule.

Much of this work has been done with the convenient protein, staphylococcal nuclease (Fig. 2 and 3), which is devoid of SS bridges and undergoes very rapid denaturation and renaturation. It may be cleaved into a variety of peptide fragments by enzymic or chemical methods (Fig. 4). The purified nuclease was fractionated by sequential immunoabsorption and elution using columns of Sepharose to which one or another of the peptide fragments had been attached. The preparation of one non-precipitating, but inactivating antibody, is summarized in Figure 5 (3). Anti-$(1\text{-}149)_N$, i.e., anti-native nuclease, was first isolated on a Sepharose-nuclease column. This mixed antibody population was then successively fractionated on Sepharose-(99-149) and Sepharose-(127-149). The "pass through" on the last column contained antibody directed against the sequence (99-126). This fraction was monodeterminant, and did not precipitate with nuclease, although it still combined with the 99-126 portion of native nuclease.

When anti-$(99\text{-}126)_N$ was added to nuclease, the enzyme was inactivated (3,4). However, addition of increasing amounts

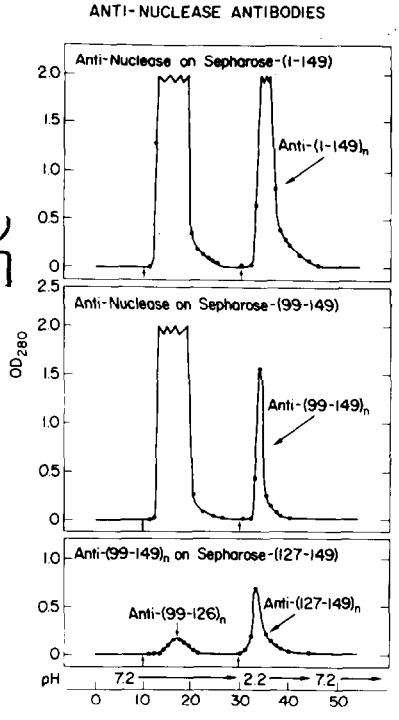

FIGURE 5. Fractionation of antibody against native nuclease by sequential absorption on Sepharose columns to which either nuclease, peptide 99-149 or peptide 127-149 have been attached.

of the randomly structured peptide 99-149 led to increasing release of the antibody-bound enzyme. Determinations of the successive activity levels permitted calculation of the equilibrium constant relating random structure, and structure sufficiently similar to the native "format" of the peptide to permit competition with nuclease for antibody $(99\text{-}126)_N$.

These calculations indicated that $(99\text{-}149)_R$ (i.e., *random*) occupied an antibody-compatible conformation, approximately 0.02% of the time. Other peptide fragments have been subjected to similar analysis and show similar propensities for assuming their native format. For example, peptide (127-149) and its monospecific $antibody_N$ were employed in an analysis similar to the above study of competition between intact nuclease and peptide fragment, and yielded the same estimate of folding frequency.

Using similar methods, one can determine the frequency of unfolding of a native structure such as nuclease (5). How

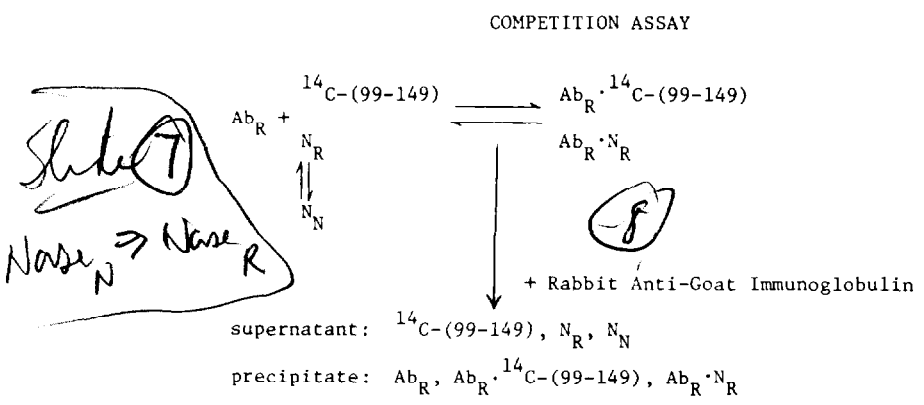

FIGURE 6. Double antibody procedure for establishing the degree to which native nuclease can unfold into a family of denatured forms.

often is a protein molecule sufficiently unwound to be recognized by an antibody directed against a random polypeptide structure? For these studies, antibody was prepared against the peptide (99-149). This antibody, $(anti\text{-}99\text{-}149)_R$, was first purified from the IgG fraction by absorption on a column of Sepharose-(99-149). The purified antibody was then reacted with ^{14}C-carbamoylated (99-149). It was found that nuclease could compete for this interaction to an extent consistent with a ^{K}conformation of 3×10^3 at 25°C. This indicates partial unfolding of nuclease. As indicated in Figure 6, the extent of competition could be measured by using rabbit anti-goat immunoglobulin to determine the fraction of the labelled peptide in the precipitate and supernatant components. When the experiments were carried out at 3°C and 30°C, the conformational equilibrium constants were 4000 and 400, respectively. Addition of stabilizing ligands for nuclease (thymidine diphosphate and calcium ions) led to a constant of 40,000.

The tendency for certain peptide fragments of protein molecules to occupy their native "format" in solution may become a strong factor in the preparation of totally synthetic vaccines. When the total sequence of a protein associated with a pathogenic agent is known, organic synthesis of a relatively small antigenic portion of the molecule may be adequate for the preparation of a useful vaccine. Such an approach has been taken by Arnon and Sela and their colleagues (6) at the Weizmann Institute of Science and, more recently, by Lerner and the synthetic vaccine group at the Scripps Clinic of the University of California in San Diego (7).

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7. This volume.

This short lecture at least not DNA recombinant - classic.
Very great pleasure to add my congratulations & wishes for the future to Anfinsen - Planning experimental program for years ahead.